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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Variation of mucin adhesion, cell surface characteristics, and molecular mechanisms among *Lactobacillus plantarum* isolated from different habitats

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Abstract The adhesion ability to mucin varied greatly among 18 *Lactobacillus plantarum* isolates depending on their isolation habitats. Such ability remained at high level even though they were sequentially exposed to the gastrointestinal (GI) stresses. The majority of *L. plantarum* isolated from shrimp intestine and about half of food isolates exhibited adhesion ability (51.06–55.04%) about the same as the well-known adhesive *L. plantarum* 299v. Interestingly, five infant isolates of CIF17A2, CIF17A4, CIF17A5, CIF17AN2, and CIF17AN8 exhibited extremely high adhesion ranging from 62.69 to 72.06%. Such highly adhesive property correlating to distinctively high cell surface hydrophobicity was significantly weaken after pretreatment with LiCl and guanidine-HCl confirming the entailment of protein moiety. Regarding the draft genome information, all molecular structures of major cell wall-anchored proteins involved in the adhesion based on *L. plantarum* WCSF1, including lp_0964, lp_1643, lp_3114, lp_2486, lp_3127, and lp_3059 orthologues were detected in all isolates. Exceptionally, the gene-trait matching between yeast agglutination assay and the relevant mannose-specific adhesin (lp_1229) encoding gene confirmed the Msa absence in five infant isolates expressed distinctively high adhesion. Interestingly, the predicted flagellin encoding genes (*fliC*) firstly revealed in lp_1643, lp_2486, and lp_3114 orthologues may potentially contribute to such highly adhesive property of these isolates.

Keywords Lactobacillus *fliC* · Mucin-adhesion mechanism · Mannose-specific adhesion · Mucus-binding protein · Gene-trait matching · Competitive adhesion

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Introduction

Lactobacillus plantarum is widely found in various habitats due to its ability to adapt and survive under many different environments. It is commonly involved in fermentation and spoilage of foods derived from many varieties of plants and animals (de Vries et al. 2006). Its ubiquitous presence is due to the ability to uptake and utilize multiple types of mono-, di-, oligo-saccharides, and peptides as well as the ability to deal with various environmental stresses according to the information acquired from the complete genome sequence of *L. plantarum* WCFS1. In addition, a large number of surface-anchored protein encoding genes confirmed the ability to adhere and then colonize on many different kinds of surfaces (Kleerebezem et al. 2003).

L. plantarum commercialized as probiotic strains must be able to colonize and persist in human guts and therefore exert various health benefits. These included reduction of pathogenic bacteria and improvement of host health from many disorders or serious diseases such as diarrhea, allergy, high blood

cholesterol, and low immunity. (Bukowska et al. 1998; Ammor et al. 2006; Zago et al. 2011). For probiotic bacteria to establish and colonize at the host target site in a substantial number necessary to continuously achieve health benefit, the ability to adhere intestinal mucosa does play a crucial contribution (Adlerberth et al. 1996; Bujalance et al. 2007; Wang et al. 2009). The mucus layer coating gastrointestinal epithelium is a continuous gel matrix composed primarily of complex glycoproteins (mucin) as a major constituent. It acts as a barrier to protect host cells from destructive antigens and also provides habitat and nutrient for gut microflora. This layer is the first physical barrier to protect the invasion of undesirable microbes to gut epithelium. Meanwhile, it serves as colonizing site for commensal microbes, which are necessary to interact with host cells for training, eliciting, or modulating host immune system. Therefore, the adhesion to mucin is required to provide substantial resident time for probiotic organisms to display their functional characteristics in host health.

The adhesion mechanism of lactobacilli involved various non-specific and specific ligand-receptor interactions. The initial mechanism was relevant to non-specific physicochemical interaction between cell surface and mucin/epithelial cells. Therefore, certain characteristics of bacterial cell surface could primarily play a role in their adhesion to the target sites (Kos et al. 2003; Schillinger et al. 2005; Ouwehand et al. 1999). Such characteristics were expressed in terms of surface charge, hydrophobicity, and electron donor-acceptor properties of bacterial cell surface. These could influence the attractive and repulsive forces between cell and mucosal surfaces, therefore modulating the adhesion level (Hamadi et al. 2012). In the meantime, the specific adhesion-receptor interaction is an essential mechanism of microbial adhesion on GI surface. Certain proteins, such as lectin-like adhesion, plays a major role in specific adhesion. This binding protein specifically target to mannose moiety within glycoconjugates molecule (mannose-specific adhesion). Its mucin binding was inhibited in the presence of methyl-α-D-mannoside (Adlerberth et al. 1996; Pretzer et al. 2005). Another proteins, such as s-layer protein (Slps) (Beck et al. 2009), glyceraldehyde-3-phosphate dehydrogenate (GAPDH), elongation factor Tu (EF-Tu), triosephosphate isomerase (TPI), mucus-binding protein (Mub) and mucus-adhesion binding protein (MapA) (Ramiah et al. 2007; Ramiah et al. 2008; Buck et al. 2005) were also involved in mucin adhesion of lactobacilli.

This study aimed to evaluate the adhesion ability of *L. plantarum* isolated from many different sources. The possible link between their mucin adhesion and sources of isolation was evaluated. The mechanisms involved in mucin adhesion, including non-specific hydrophobic and specific adhesion-receptor interactions, were characterized. The bacterial cell surface properties dealing with adhesion ability was determined after the pretreatment with protease enzymes and certain chemicals. The mannose-specific adhesion of *L. plantarum* was also determined using yeast agglutination assay. Moreover, the genes relevant to mucin adhesion, including mannose-specific adhesion (*msa*) and mucus-binding proteins (*mup*), were characterized and compared to reveal molecular mechanisms through gene-trait matching between phenotypic and genotypic characteristics. The latter could be drawn from the genome sequences based on the complete whole genome of *L. plantarum* WCSF1 to locate, identify, and predict certain genes encoding cell surface proteins relating to mucin adhesion.

Materials and methods

Bacterial strains and culture conditions

Eighteen strains of *L. plantarum* selected based on their distinctive antimicrobial activity were isolated from many different sources. These included five strains of CIF17A2, CIF17A4, CIF17A5, CIF17AN2, and CIF17AN8 from infant feces (Uraipan and Hongpattarakere 2015); four strains of MHO2.4, MHO2.5, MHO2.9, and A3 from shrimp intestine (Hongpattarakere et al. 2012; Kongnum and Hongpattarakere 2012). Additionally, nine strains of K35, K36, T10, P14, P22, P42, P62, P76, and P86 isolated from traditional fermented fish and rice products were provided by A. Nuylert and K. Kanjanasmith, Department of Industrial Biotechnology, Prince of Songkla University (Songkhla, Thailand). Those were deposited at Thailand Institute of Scientific Technology Research (TISTR) Culture Collection as TISTR numbers 2488, 2489, 2490, 2491, 2492, 2485, 2486, 2487, 2472, 2469, 2470, 2475, 2476, 2477, 2480, 2481, 2484, and 2471, respectively. The well-known adhesive strain of *L. plantarum* 299v (DSM 9843) was provided by Assoc. Prof. Dr. Sunee Nitisinprasert, Kasetsart University, Thailand. *Escherichia coli* O157:H7 DMST 12743 was obtained from the Department of Medical Science, Ministry of Public Health (Bangkok, Thailand). *Escherichia coli* TISTR 780 and *Staphylococcus aureus* TISTR 1466 were received from TISTR, Bangkok, Thailand. All patient isolates of *Salmonella enterica* subsp. *enterica* serovar Thyphimurium SA2093 and *Shigella sonnei* were obtained from Microbiological Laboratory of Songklanagarind Hospital, Prince of Songkla University (Songkhla, Thailand). All strains of *L. plantarum* were cultivated in de Man Rogosa and Sharpe (MRS) broth (Himedia, India) at 37 °C for 18 h. The pathogenic bacteria were grown in Mueller Hinton Broth (Himedia, India) for at 37 °C for 15 h. All bacteria were maintained and stored at – 28 °C in 30% glycerol.

Mucin adhesion and competitive adhesion of *L. plantarum* against foodborne pathogenic bacteria

The adhesion assay was performed according to Uraipan et al. (2014). The partially purified porcine gastric mucin type III

(Sigma, USA) was coated on a sterilized polystyrene microtiter plate well (Maxisorp Nunc, Denmark). A minimum of four replicates was used to estimate the adhesion of the tested strain. *Lactobacillus plantarum* 299 V, a well-known mucin adhesive strain, was used as a positive control. Briefly, 100 μL of probiotic culture (1×10^7 CFU/mL) was added to each mucin-coated well before incubation at 37 °C for 1 h. Each well was thoroughly washed twice with 200 μL sterile phosphate buffered saline (PBS) to remove unbound bacteria before 200 μL of a 0.05% (*v*/*v*) Triton X-100 solution (Sigma-Aldrich, Singapore) were added. The plates were then incubated at ambient temperature for 2 h under gentle agitation to dislodge the bound bacteria (N_{Adhere}). The viable cells of *L. plantarum* were enumerated before ($N_{Initial}$) and after (N_{Adhere}) mucin adhesion by plating the appropriate dilution on MRS agar. The percentage of adhesion was calculated according to (log CFU N_{Adhere}/logCFU $N_{Initial}$) × 100.

The ability of *L. plantarum* to competitively exclude many foodborne pathogenic bacteria for mucin adhesion was determined using competition and displacement assays. Both assays were performed according to Uraipan et al. (2014). For the first assay, 50 μL of *L. plantarum* and pathogenic bacteria (approximately 5×10^5 CFU of each) were mixed and then added to a mucin-coated well. In displacement assays, the pathogen was firstly allowed to adhere for 1 h and *L. plantarum* was subsequently introduced. After incubation at 37 °C for 1 h, the unbound bacteria were removed by washing twice with PBS buffer. The adhered pathogen was detached by treating with 200 μL of 0.05% (*v*/*v*) Triton X-100 solution for 2 h under orbital agitation at ambient temperature before being enumerated on MHA, in which *L. plantarum* could not develop any colony. The adhesion percentages of the pathogen in both treatments (with and without *L. plantarum*) were calculated as mentioned above. The competitive adhesion of *L. plantarum* against pathogenic bacteria was expressed and calculated from the differences between adhesion percentages of pathogens in the absence and presence of *L. plantarum*.

Adhesion ability of *L. plantarum* after exposure to gastrointestinal stresses

The adhesion percentage of *L. plantarum* was determined before and after exposure to the mimicked GI stresses. The experiment was performed based on Vizoso Pinto et al. (2006) and Mathara et al. (2008) with some modification. To expose *L. plantarum* to the simulated GI transit condition prior adhesion assay, the cell pellet was resuspended and incubated in sterile electrolyte solution (SES; 0.22 g/L $CaCl_2$, 6.2 g/L NaCl, 2.2 g/L KCl, 1.2 g/L $NaHCO_3$) containing 100 mg/L of lysozyme for 5 min at 37 °C. The mixture was then centrifuged at 6000×*g* 15 min, and the pellet was resuspended in an artificial gastric fluid (pH 2.5) comprising of 6.2 g/L NaCl, 2.2 g/L KCl, 0.22 g/L $CaCl_2$, 1.2 g/L $NaHCO_3$, and 3.0 g/L pepsin (Sigma, Germany). After 1 h of incubation at 37 °C, the cells were centrifuged and incubated in sterile PBS (pH 8.0) containing 0.1% (*w*/*v*) pancreatin from porcine pancreas (Sigma, Germany) and 0.3% (*w*/*v*) oxgall bile salt (Merck, Germany) at 37 °C for 3 h. The cells were then centrifuged and washed twice with sterile PBS (pH 7.0) before the viable cells were enumerated on MRS agar. All treated and control (exposed to 0.85% NaCl) cells were subjected to adhesion assay described above.

Physicochemical characteristics of *L. plantarum* cell surface

Microbial adhesion to solvents

Microbial adhesion to organic solvents (MATS) was measured according to Kos et al. (2003) with some modifications. Three different solvents, including xylene, chloroform, and ethyl acetate which represented either non-polar (hydrophobicity), monopolar and acidic (electron donor), or monopolar and basic (electron acceptor), respectively were used in this phase partition assay. Bacteria were harvested by centrifugation at 6000×*g* for 15 min and washed twice with sterile PBS, pH 7.0. After that, the pellet was resuspended and diluted in the same buffer to approximately 1×10^8 CFU/mL. The absorbance of the cell suspension was measured at 600 nm (A_0). One milliliter of solvent was added to 1 mL of cell suspension. The two-phase system was thoroughly mixed by vortexing for 2 min. The aqueous phase was removed after 20 min of incubation at room temperature, and its absorbance at 600 nm (A_1) was measured. The percentage of microbial adhesion to solvent was calculated from % MATE = $[(1-A_1)/A_0] \times 100$.

Enzymatic and chemical pretreatments of bacterial cells

This experiment was carried out according to Tallon et al. (2007) and Ramiah et al. (2008) with some modification. *L. plantarum* harvested at late log phase (18 h incubation) was washed twice with sterile PBS (pH 7.0) before treated with either 2 mg/mL of trypsin (Sigma, USA) at 37 °C for 1 h or 4 mol/L guanidine-HCl (Amresco, USA) or 5 mol/L LiCl for 30 min at 4 °C with gentle agitation. The cells treated under each condition were then washed twice with PBS pH 7.0 and resuspended in PBS to achieve a final concentration of 1×10^7 CFU/mL for subjecting to adhesion assay. Bacteria exposed to PBS were used as a control. The adhesion percentage was calculated according to (log CFU N_1/log CFU N_0) × 100, where N_0 and N_1 are the number of bacteria before and after the pretreatment, respectively.

Autoaggregation and coaggregation assays

The ability of *L. plantarum* to autoaggregate and coaggregate *E. coli* O157:H7 were determined based on Kos et al. (2003) with some modifications. Bacteria cultivated according to the above condition were washed and resuspended in PBS to achieve viable counts about 10^8 CFU/mL. For autoaggregation assay, the cell suspension of *L. plantarum* was vortexed for 10 s. The tube was then incubated at room temperature for 4 h to allow cell aggregating. The autoaggregation of *L. plantarum* was determined by measuring the absorbance at 600 nm of the upper layer. The aggregation percentage was calculated according to $1 - (A_1/A_0) \times 100$, where A_0 and A_1 represented the absorbance at time 0 and 4 h, respectively. The coaggregation was performed in a similar manner, in which equal volumes of cell suspension from *L. plantarum* and *E. coli* O157:H7 were mixed together by vortexing for 10 s. The tube was incubated for 4 h before the absorbance of the upper layer was measured.

Yeast agglutination assay

The mannose-specific adhesion of *L. plantarum* was determined using yeast agglutination assay (Pretzer et al. 2005). In brief, *L. plantarum* culture (1 mL) was washed twice with sterile PBS (pH 7.4) and then diluted in the same buffer to achieve final concentration of 1×10^9 CFU/mL. Fifty microliters of the bacterial suspension were transferred into each well of a 96-well microtiterplate before 50 μL of either PBS (control) or 25 mM of methyl-α-D-mannopyranoside (Sigma, USA) containing PBS was added. One hundred microliters of *Saccharomyces cerevisiae* (1×10^9 cells) was added before the microtiter plate was shaken for 10 min at ambient temperature. The sample (30 μL) was taken from each well to examine agglutination of yeast cells under a bright-light microscope (200-fold magnification; Zeiss, Axio scope A1, Germany). The ability of *L. plantarum* to induce visible yeast agglutination was observed and performed in three independent experiments.

Genomic sequences based on genes involved in adhesion

The genomic DNA was extracted from 1.5 mL overnight culture of all *L. plantarum* isolates using commercial kit MasterPure™ (Epicenter, Madison, USA). The whole genome was then sequenced on a paired-end (R1 = 326 bp, R2 = 286 bp) Illumina MiSeq run at the DNA sequencing and genomics laboratory, Institute of Biotechnology, University of Helsinki. Adapter sequences and low-quality bases ($Q < 30$) at the ends of reads were trimmed out using cutadapt (version 1.6) (Martin 2011). Overlapping paired-end reads were merged and extended as single reads by FLASH (Fast Length Adjustment of SHort reads) (version 1.2.6) (Magoč and Salzberg 2011). De novo genome assemblies were performed from the extended paired-end and orphan reads using SPAdes assembler (version 3.1.1) (Bankevich et al. 2012). Gene prediction and annotation were done using the RAST server (Aziz et al. 2008). The analyzed whole genome shotgun sequences of *L. plantarum* strains of CIF17A2, CIF17A4, CIF17A5, CIF17AN2, CIF17AN8, MHO2.4, MHO2.5, MHO2.9, A3, K35, K36, T10, P14, P22, P42, P62, P76, and P86 were deposited in GenBank under accession numbers of LEBD00000000, LEBC00000000, LEBB00000000, LEBA00000000, LEAZ00000000, LEBI00000000, LEBH00000000, LEBG00000000, LEBF00000000, LEBT00000000, LEBW00000000, LEBS00000000, LEBV00000000, LEBR00000000, LEBO00000000, LEBN00000000 LEBK00000000, and LEBJ00000000, respectively.

The presence of LPXTG cell wall-anchored *mub* and *msa* homologs was searched within the draft genome sequences of all isolates. They were blasted for mucus-binding proteins according to lp_1643, lp_3114, lp_0964, lp_3127, lp_2486, lp_3059, and lp_1229 loci of *L. plantarum* WCSF1 using a two-sequence alignment of NCBI blast program (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch&BLASTSPEC=blast2seq&LINK_LOC = align2seq). The MEGA 6.06 program with *p* distance method was applied for analyzing nucleotide and protein sequence similarity of these gene loci shared among all isolates of *L. plantarum*. The structural domains organized within each locus were elucidated and identified using Hidden Markov model (HMM) and NCBI conserves domain database (CDD) (Marchler-Bauer et al. 2015). The protein sequences were deduced, and their adhesion role was then predicted. Search for proteins carrying MucBP domains was confirmed using Pfam model (alignment model, PF06458).

Statistical analysis

Significant mean differences existed between strains and parameters tested were analyzed using one-way analysis of variance and Duncan's multiple-range test. The statistical difference was indicated at $P < 0.05$. Each data set was obtained from four replicates.

Results

Mucin adhesion of *L. plantarum* isolated from many different habitats

Eighteen isolates of *L. plantarum* obtained from different sources revealed a wide variation of adhesion ability to mucin (Table 1). The extraordinarily high level of mucin adhesion was confined among all isolates derived from infant feces,

Table 1 Mucin adhesion, cell surface property, yeast agglutination, coaggregation with *E. coli* O157:H7, and competitive adhesion (exclusion and displacement) against food-borne pathogenic bacteria of *L. plantarum* isolates

Strains	Adhesion (%)	% MATE	Agglutination assay	Coaggregation (with *E. coli* O157:H7)	% Competitive exclusion, mean ± SD#		
					E. coli TISTR780	*E. coli* O157:H7	*Sal.* Typhimurium
K35	45.4 ± 4.6^{f}	27.5 ± 5.6^{f}	+	10.3 ± 0.5^{ef}	− 0.29 ± 1.8	1.3 ± 1.2	− 0.90 ± 1.0
K36	52.6 ± 3.4^{def}	54.3 ± 5.4^{cd}	+	9.2 ± 1.3^{f}	− 3.32 ± 1.4	20.7 ± 1.1*	2.0 ± 1.8
T10	34.1 ± 1.0^{g}	N	N	10.5 ± 0.0^{ef}	N	N	N
P14	32.5 ± 2.2^{g}	N	N	11.5 ± 0.4^{de}	N	N	N
P22	46.2 ± 7.0^{def}	57.7 ± 7.1^{c}	+	10.8 ± 0.1^{ef}	0.0 ± 4.5	22.4 ± 7.0*	0.9 ± 2.1
P42	52.7 ± 3.2^{def}	12.4 ± 4.2^{g}	+	13.7 ± 0.6^{c}	− 2.4 ± 2.2	16.0 ± 4.4*	5.2 ± 3.1
P62	55.0 ± 1.4^{d}	10.2 ± 3.8^{g}	+	11.6 ± 0.1^{de}	10.0 ± 3.9*	21.6 ± 5.0*	2.6 ± 0.4
P76	35.2 ± 2.2^{g}	N	N	10.7 ± 0.6^{ef}	N	N	N
P86	51.7 ± 0.2^{e}	37.0 ± 8.2^{ef}	+	11.1 ± 0.9^{e}	0.3 ± 6.5	14.5 ± 2.6*	5.4 ± 1.5*
MHO 2.4	50.5 ± 3.8^{def}	27.5 ± 5.0	+	13.2 ± 0.7^{c}	0.6 ± 5.7	23.0 ± 1.4*	4.4 ± 0.4*
MHO 2.5	47.5 ± 2.8^{f}	45.9 ± 7.9^{de}	–	12.9 ± 1.1^{cd}	− 3.2 ± 0.5	6.0 ± 1.3*	4.3 ± 1.0*
MHO2.9	53.1 ± 2.1^{de}	56.0 ± 6.1^{cd}	–	14.1 ± 0.6^{c}	− 0.4 ± 3.5	12.7 ± 0.4*	4.0 ± 2.8
A3	51.1 ± 2.7^{de}	57.3 ± 4.1^{c}	+	13.5 ± 0.6^{c}	− 2.7 ± 1.4	1.7 ± 2.2	5.0 ± 1.0*
CIF17A2	68.9 ± 5.4^{abc}	75.1 ± 5.2^{ab}	–	15.7 ± 1.7^{b}	0.4 ± 2.0	21.1 ± 3.5*	4.4 ± 0.9*
CIF17A4	62.7 ± 1.7^{c}	80.9 ± 6.8^{a}	–	20.1 ± 1.0 ^{a}	− 3.7 ± 1.2	21.7 ± 3.2*	4.1 ± 2.0
CIF17A5	70.8 ± 1.6^{ab}	67.8 ± 10.4^{bc}	–	20.4 ± 0.8^{a}	7.0 ± 0.9*	2.9 ± 2.4	3.9 ± 0.9*
CIF17AN2	67.0 ± 2.6^{b}	65.7 ± 8.3^{bc}	–	16.5 ± 1.4^{b}	8.3 ± 1.4*	7.8 ± 3.3*	4.5 ± 1.0*
CIF17AN8	72.1 ± 0.4^{a}	71.1 ± 3.9^{ab}	–	17.0 ± 1.5^{b}	− 4.7 ± 1.0	14.9 ± 4.5*	3.0 ± 1.3
299 V	55.5 ± 3.5^{d}	71.7 ± 1.3^{ab}	+	12.9 ± 0.6^{cd}	− 0.4 ± 3.3	16.3 ± 1.0*	1.9 ± 4.7

Strains	% MATE	% Competitive exclusion, mean ± SD#		% Displacement, mean ± SD ##	
		S. aureus	*Shi. sonnei*	*S. aureus*	*Shi. sonnei*
K35	27.5 ± 5.6^{f}	10.4 ± 3.2*	6.3 ± 1.8*	5.8 ± 2.0*	7.6 ± 1.7*
K36	54.3 ± 5.4^{cd}	1.0 ± 2.2	− 1.8 ± 2.5	3.9 ± 2.1	12.3 ± 2.9*
T10	N	N	N	N	N
P14	N	N	N	N	N
P22	57.7 ± 7.1^{c}	− 3.0 ± 3.0	− 0.9 ± 2.5	− 4.0 ± 4.0	9.8 ± 4.1*
P42	12.4 ± 4.2^{g}	− 2.6 ± 0.8	− 9.6 ± 0.4*	− 3.2 ± 2.7	15.7 ± 3.5*
P62	10.2 ± 3.8^{g}	− 1.2 ± 1.5	3.7 ± 3.4	− 4.1 ± 2.9	15.1 ± 3.7*
P76	N	N	N	N	N
P86	37.0 ± 8.2^{ef}	− 0.1 ± 0.6	− 4.3 ± 2.9	− 2.7 ± 4.4	13.2 ± 3.6*
MHO 2.4	27.5 ± 5.0	1.9 ± 2.2	− 5.8 ± 2.8	0.9 ± 4.1	16.2 ± 2.8*
MHO 2.5	45.9 ± 7.9^{de}	15.1 ± 4.2*	5.7 ± 2.3	− 1.4 ± 1.9	7.9 ± 1.5*
MHO2.9	56.0 ± 6.1^{cd}	1.85 ± 0.7	− 7.0 ± 1.0*	2.2 ± 2.0	9.7 ± 2.8*
A3	57.3 ± 4.1^{c}	11.4 ± 1.9*	6.8 ± 2.7*	5.4 ± 1.2*	13.4 ± 4.7*
CIF17A2	75.1 ± 5.2^{ab}	1.8 ± 0.3	− 4.1 ± 2.2	− 3.3 ± 3.3	8.7 ± 2.0*
CIF17A4	80.9 ± 6.8^{a}	− 1.2 ± 1.50	− 3.5 ± 2.7	3.2 ± 2.9	15.1 ± 6.2*
CIF17A5	67.8 ± 10.4^{bc}	11.0 ± 2.4*	4.0 ± 0.4*	− 4.2 ± 2.7	0.5 ± 3.1
CIF17AN2	65.7 ± 8.3^{bc}	12.5 ± 3.1*	2.5 ± 1.9	6.9 ± 1.2*	4.2 ± 2.2
CIF17AN8	71.1 ± 3.9^{ab}	0.1 ± 1.6	− 5.1 ± 1.2	− 3.5 ± 3.1	13.0 ± 2.0*
299 V	71.7 ± 1.3^{ab}	− 1.2 ± 2.9	− 4.1 ± 0.9	− 4.3 ± 5.5	14.0 ± 4.9*

The single number sign indicates competitive exclusion, and the double number sign indicates displacement of *L. plantarum* against pathogenic bacteria. A high value represents a low number of adhered pathogens in the presence of *L. plantarum* when compared to the adhesion of pathogen alone. A low value represents a high number of adhered pathogens in the presence of *L. plantarum* when compared to adhesion of the pathogen alone

N not tested, + positive detection, − negative detection

*Significant difference ($P < 0.05$) compared with pathogen alone as a control

[a-f] Different superscripted letters indicate significant differences (P < 0.05) within the same column

which were much greater than the well-known adhesive *L. plantarum* 299v and all tested isolates. Overall, *L. plantarum* isolated from traditional fermented foods and shrimp intestine showed lower adhesion level with high variation. The three strains of *L. plantarum* T10, P14, and P76 with much lower adhesion level than the control strain were then excluded from further study.

Influences of gastrointestinal stresses on adhesion ability of *L. plantarum*

Among 15 strains tested, the adhesion ability of nine strains significantly ($P < 0.05$) reduced after exposure to the simulated saliva, gastric, and intestinal stresses when compared with non-treated ones. The average adhesion ability was reduced ranging from 6.02 to 20.88% after pretreated with lysozyme and intestinal fluid (Fig. 1a). The slight reduction was observed in a few of isolates derived from fermented food (P22 and P86), shrimp intestine (MHO 2.9), and infant feces (CIF17A2 and CIF17AN8). Nevertheless, the adhesion of all isolates still remained at the level as high as the control strain even thought they were exposed to the extreme GI stresses.

Competitive adhesion of *L. plantarum* against foodborne pathogenic bacteria

The competitive adhesion of *L. plantarum* against different foodborne pathogenic bacteria was observed thorough competitive adhesion and displacement assays. In the first assay, *L plantarum* and pathogenic bacteria were simultaneously introduced to competitively adhere to mucin, whereas, the pathogen was firstly allowed for mucin binding in the latter one. Interestingly, all strains of *L. plantarum* significantly outcompeted *E. coli* 0157:H7 in both exclusion and displacement

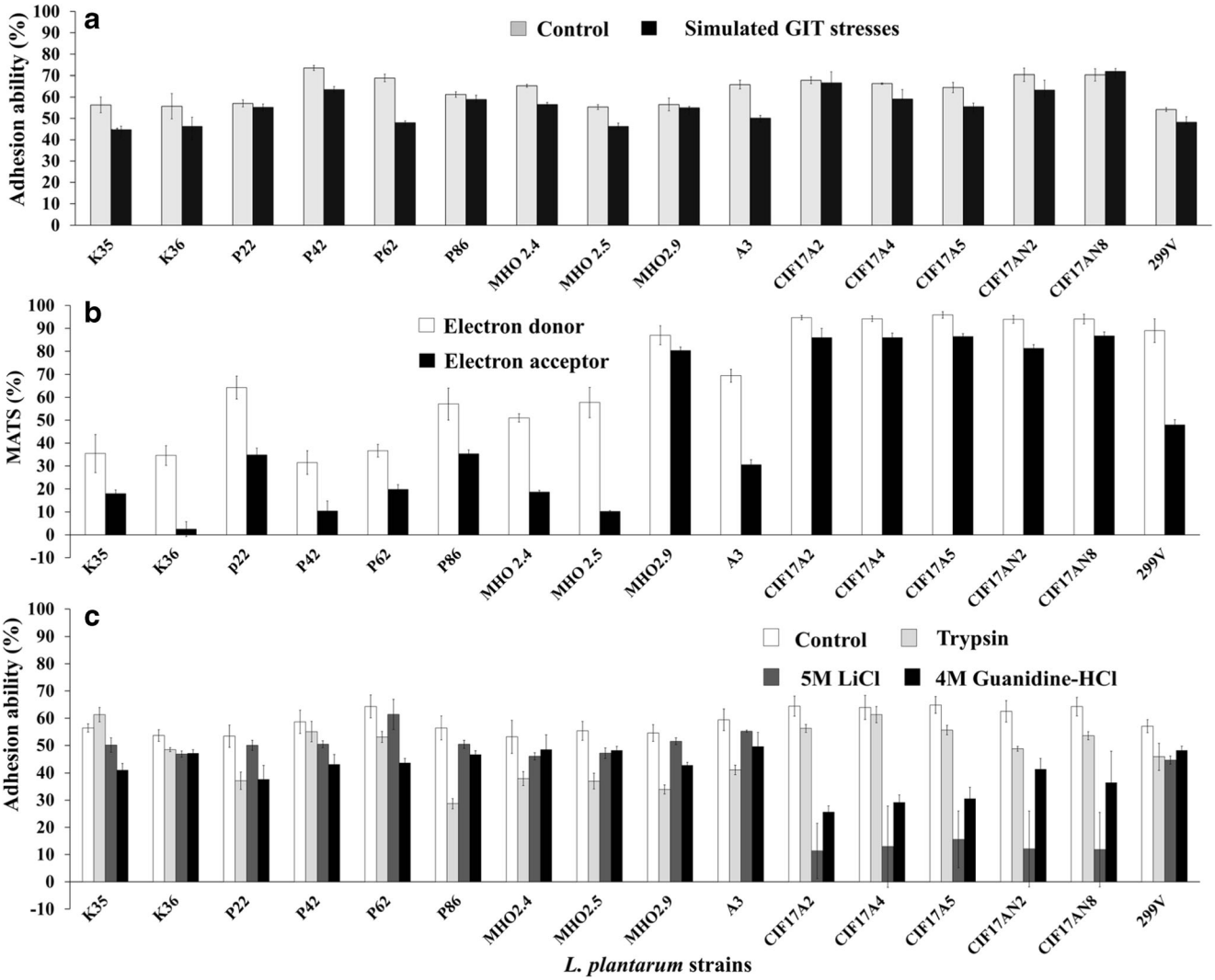

Fig. 1 Adhesion ability of *L. plantarum* after exposures to (**a**) GIT stresses, (**b**) chloroform and ethyl acetate partition assay, and (**c**) trypsin and protein denaturants (5 M LiCl and 4 M guanidine-HCl) compared with non-treated one as a control

assays as shown in Table 1. However, only three strains of *L. plantarum* (P42, CIF17A5, and CIF17AN2) were able to thrive ($P < 0.05$) over *E. coli* TISTR 780 in the competitive adhesion. The majority of *L. plantarum* overcame *Sal. enterica* subsp. *enterica* serovar Typhimurium for mucin binding in both assays. Most *L. plantarum* (except CIF17A5 and CIF17AN2) were able to displace *Shig. sonnei* significantly with the values ranged from 7 to 17%. On the contrary, *Shig. sonnei* could overcome most *L. plantarum* in the competitive assay.

Physiochemical and physiological properties of *L. plantarum* cell surface

The adhesion mechanisms of *L. plantarum* isolates were explored through several chemical and enzymatic pretreatments. MATE method was used to evaluate the hydrophobic/hydrophilic cell surface properties of *L. plantarum*. The wide variation of cell partition percentage to xylene reflected great difference of cell surface hydrophobicity among all *L. plantarum* isolates. The extraordinarily high hydrophobicity was clearly observed in five infant isolates (CIF17A4, CIF17A2, CIF17A5, CIF17AN8, and CIF17AN2) with percentage of MATE ranging from 65 to 80% (Table 1). This was well correlated to their greatest levels of autoaggregation (data not shown) and coaggregation with *E. coli* O157:H7 as shown in Table 1.

In addition to hydrophobic cell surface, bacterial partition to chloroform and ethyl acetate was performed to assess the electron donor-acceptor (Lewis acid-base) characteristic of bacterial cell surface. Six strains (K35, K36, P22, MHO2.4, MHO2.5, A3, and 299 V) preferred ethyl acetate to chloroform phase indicating electron donor molecules were abundantly present on cell surface (Fig. 1b). While cell surface characteristics of six strains (MHO2.9, CIF17A2, CIF17A4, CIF17A5, CIF17AN2, and CIF17AN8) exhibited strong affinity to both chloroform and ethyl acetate, the result supported variability of cell surface molecules among *L. plantarum* isolates originated from many different sources.

Moreover, many protein denaturant pretreatments confirmed the diversity of cell surface properties within *L. plantarum*, which responded to trypsin, LiCl, and guanidine-HCl at various degree. Interestingly, mucin adhesion of all infant fecal isolates was strongly abolished after pretreatment with strong protein denaturants, such as LiCl and guanidine-HCl, whereas, the majority of *L. plantarum* derived from other sources slightly responded (Fig. 1c). This suggested that cell surface proteins could play a key role in mucin adhesion of infant isolates of *L. plantarum*, which was further proven by testing for specific adhesion-receptor interaction using yeast agglutination model.

All food isolates were able to agglutinate *S. cerevisiae* cells, of which mannose-containing polysaccharides were abundantly present in the cell wall. The yeast agglutination of these isolates was dramatically inhibited in the presence of methyl-α-D-mannoside (Fig. 2). The results supported that the adhesion specific for mannose-containing receptors strongly involved in mucin adhesion of these food-derived isolates. Distinctively, most infant and half of shrimp isolates showed a negative response in yeast agglutination assay implying that the other adhesion mechanisms were predominantly responsible for mucin adhesion in human and shrimp hosts.

Mucin adhesion protein encoding genes involved in adhesion of *L. plantarum*

The genes responsible for mucin adhesion based on loci of lp_1643, lp_3114, lp_0964, lp_3127, lp_2486, and lp_3059 of *L. plantarum* WCSF1 were predicted within the genomes of all tested isolates. On the other hand, lp_1229 (*msa*) was found in draft genome sequences of 11 isolates correlating to their positive responses in yeast agglutination assay as reported in Table 1. All predicted surface proteins contained N-terminal leading and C-terminally LPXTG-anchored sequences. The identity percentage of amino sequences deduced from their *msa* genes ranging from 89 to 99% in comparison with *L. plantarum* WCSF1. Surprisingly, *msa* was absent in five infant and two shrimp isolates (CIF17A4, CIF17A2, CIF17A5, CIF17AN8, CIF17AN2, MHO2.5, and MHO2.9) as shown in Fig. 3g. This evidence indicated that the adhesion mechanism mediated through mannose-specific protein interaction played no role in mucin adhesion of these intestinal isolates.

According to domain organization identified by Hidden Markov model (HMM) and NCBI conserve domain database (CDD) (Marchler-Bauer et al. 2015), the proteins adhering to mucus shared common characteristics of cell surface proteins of Gram-positive bacteria. These included signal peptide, C-terminal cell wall anchoring motif like LPXTG, repeated domains with a putative function, and zones of unknown function. The number variation of MucBP repeated domains was observed within these gene sequences based on lp_1643, lp_3114, lp_0964, lp_3127, lp_2486, and lp_3059 depending on isolates and gene loci (Fig. 3). The repeating numbers of binding domains (MucBP) were outstandingly high in lp_0964 locus of five infant isolates (Fig. 3a). Interestingly, the lp_3114 and lp_2486 orthologues of such infant isolates shared a low similarity in nucleotide and protein sequences as low as 65–75 and 57–64%, respectively when compared to most isolates. Meanwhile, high levels of similarity (94–100%) were observed among lp_0964, lp_3059, and lp_3127 of all isolates.

Surprisingly, FliC domain was also predicted on lp_3114, lp_2486, and lp_1643 loci of these infant isolates (Fig. 3b–d). These domains related to the flagellin proteins with 132, 145, and 96 amino acids in length, respectively. It was located in

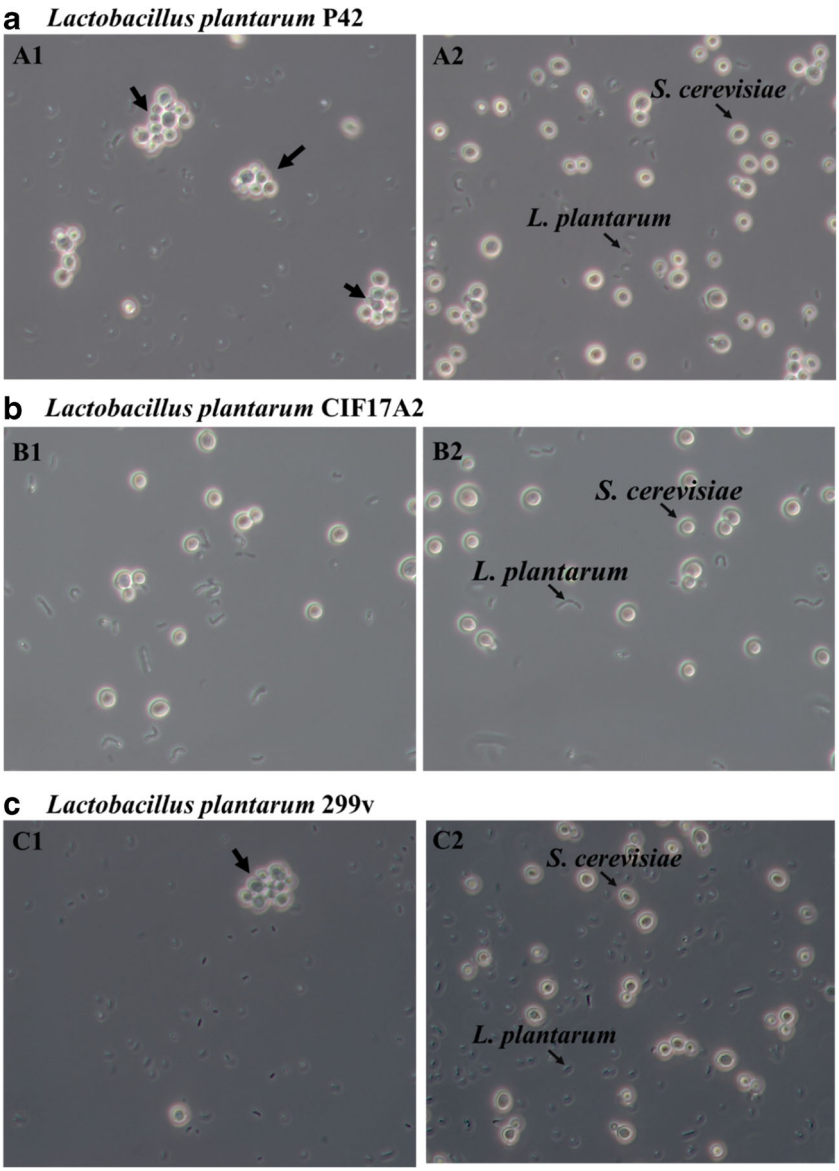

Fig. 2 Agglutination of *Saccharomyces cerevisiae* mediated by mannose-specific adhering protein of *Lactobacillus plantarum* (×10 dilution) observed under bright-light microscopy (200-fold magnification) in the absence (A1, B1, C1) and presence (A2, B2, C2) of methyl-α-D-mannoside

association with pfam00669 and pfam00700, which may involve in bacterial adhesion (Kwang et al. 1996). FliC predicted domains located within lp_3114 and lp1643 orthologues were matched with a non-specific hit of Flagellin_N, when they were aligned with well-known flagellin (available in NCBI) using Bioedit program. In the meantime, such domain of lp_2486 was matched with a specific hit of PRK08026 protein domain and Flagellin_C superfamily with an *E* value of $1.04e^{-03}$.

Discussions

The adhesion to host mucosal surface is a prerequisite to ensure probiotic colonization and persistence in the host gut at a substantial number required for many health-promoting effects. The mucus layer is not only the first physical barrier to protect gut epithelium from bacterial invasion but also the first site for gut bacteria to interact with host cells. This study revealed the high variation of adhesion ability among *L. plantarum* depending on strains and ecological origin as previously confirmed by many researchers (Adlerberth et al. 1996; Duary et al. 2011; Pretzer et al. 2005; Rinkinen et al. 2003; Tallon et al. 2007). Interestingly, the distinctively high adhesion was observed among infant isolates. This reflected the bacterial adaptation for the successful establishment in the host gut, at which the effective attachment to the mucosal surface was strongly required in order not to be swept by peristalsis.

All isolates used in this study behaved differently in mucin adhesion when exposed to gut stresses and various chemical/enzymatic pretreatments suggesting the involvement of many

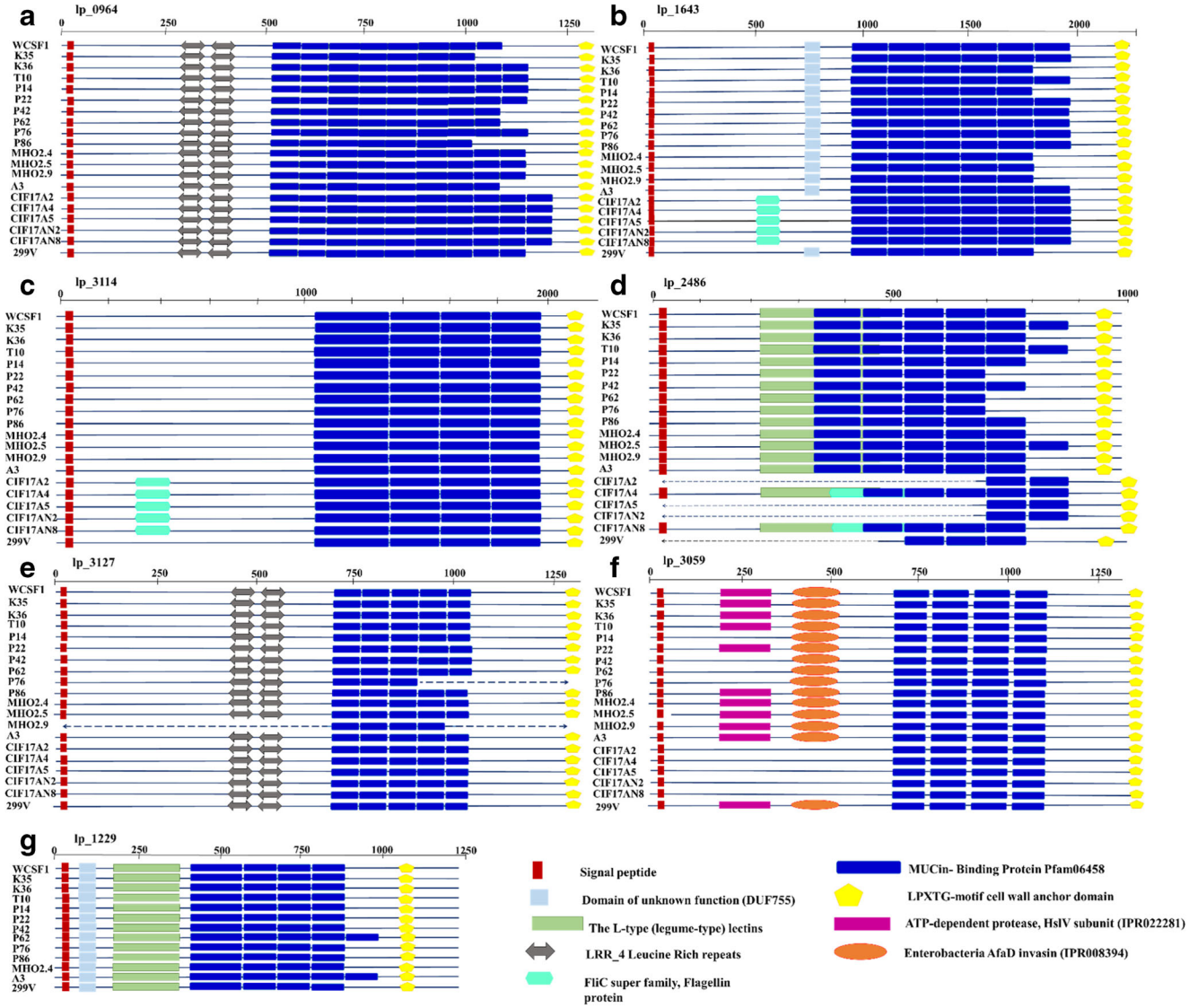

Fig. 3 Domain organization within the Msa protein like adhesin (lp_1229) and mucus-binding protein (lp_1643, lp_3114, lp_0964, lp_3127, lp_2486, lp_3059) according to HMM and CDD search

different adhesion mechanisms and high heterogeneity of cell wall components among *L. plantarum*. Such variation could influence the level and type of cell surface interactions, including electrostatic/charged, hydrophobic/hydrophilic, and receptor/protein specific binding interactions. In the presence of protein denaturants like LiCl and guanidine-HCl, severe impairment of mucin adhesion was particularly observed among the highly adhesive infant isolates. At high concentration, guanidine-HCl could mask the electrostatic interaction present in the protein molecules due to its high ionic strength and the attachment of Gdn^+ and Cl^- ions to the charged amino acid side chain of cell surface proteins (Monera et al. 1994). The chaotropic lithium ion could cause the destruction of water structure and reduction of hydrophobic interaction. These results corresponded to MATS assay, in which these isolates exhibited strong affinity to both electron donor and acceptor solvents, apart from expressing high hydrophobicity. These suggested the adhesion mechanisms were dominantly mediated through electrostatic and hydrophobic interactions. Such cell surface characteristics were obviously correlated to the remarkable ability in coaggregation with *E. coli* O157:H7 as well as the ability to exclude and compete against many foodborne pathogenic bacteria of these infant isolates.

The relationship between cell hydrophobicity and pathogen aggregation was also noted in *Lactobacillus acidophilus* M92 (Kos et al. 2003). The positive correlation between adhesion ability and surface hydrophobicity was similarly reported in bifidobacteria (Rahman et al. 2008) and human fecal *L. plantarum* (Yadav et al. 2013). However, no significant correlation between hydrophobicity and adhesion ability of lactobacillus was noted in a certain study (García-Cayuela et al. 2014). This was also similar to what was noted in

food-derived *L. plantarum* P42 and P62 in this study. Such conflict could be attributed to the variation and difference of expression level and/or types of cell surface proteins among *L. plantarum* strains. In many previous studies, the major molecules responsible for lactobacillus adhesion to intestinal epithelium and/or mucin were extracellular/transmembrane protein moieties (Båth et al. 2005; Pretzer et al. 2005; Tallon et al. 2007). Many different adhesion proteins and mechanisms of lactobacilli were clearly revealed and identified. These included 29 kDa collagen-binding protein (CnBP) of *Lactobacillus reuteri* (Roos et al. 1996), mucus-binding proteins (Mub) of *L. reuteri* 1063 (Roos and Jonsson 2002), lectin-like mannose-specific adhesin (Msa) of *L. plantarum* (Pretzer et al. 2005), mucus-binding pilin (SpaC) of *Lactobacillus rhamnosus* GG and cell wall-anchored protein CwaA of *L. plantarum* NL42 (Zhang et al. 2015).

In this study, the mannose-specific adhesion interaction, which was a common mechanism shared among *L. plantarum* (Pretzer et al. 2005), was detected and identified in all food isolates by gene-trait matching between yeast agglutination assay and genotypic information regarding the relevant *msa* gene. The complete loss of yeast agglutination activity in the presence of mannose analogue was revealed in corresponding to the presence of *msa* (lp_1229) in their genomes. However, this particular gene was absent in five infant isolates and two intestinal shrimp isolates correlating to their lack of yeast agglutination activity. The similar relevance was previously confirmed by the complete loss of yeast agglutination in a mutant of *L. plantarum* WCFS1, of which lp_1229 gene was deleted (Pretzer et al. 2005). Thus, the mannose-specific interaction had no involvement in extremely high adhesion and coaggregation with *E. coli* O157:H7of these infant derived *L. plantarum*.

Apart from the absence of *msa* gene, these infant isolates exhibited high distinction of genes responsible for mucin adhesion based on lp_0964, lp_2486, and lp_3114 loci of *L. plantarum* WCSF1. The highest number of repeated MucBP domain (pfam06458) within the lp_0964 orthologue was observed in five infant isolates displaying extremely high adhesion to mucin. The MucBP domain was generally found within MUB like adhesin based on lp_1643, lp_3114, lp_0964, lp_3127, and lp_3059 loci of *L. plantarum* WCSF1. The number variation of repeated MucBP domains within the same orthologue was previously proven to improve mucin-binding capacity. The MucBP domain accommodating proteins were previously suggested to play a potential role in host-microbe interaction in the gut (Boekhorst et al. 2006; Kleerebezem et al. 2003). Nevertheless, such relevance was not observed in other loci of all isolates used in this study.

Interestingly, FliC domain function was included in lp_1643 and lp_3114 orthologues of the five infant isolates. Several researchers reported that FliC, the major constituent of flagella, essentially involved in mucin adhesion of many pathogenic bacteria and a few species of probiotic lactobacilli (Haiko and Westerlund-Wikström 2013; Mahaja et al. 2009; Tasteyre et al. 2001; Kajikawa et al. 2011). Flagella-mediated adherence to host mucus could be a crucial prerequisite to achieve mucosal colonization of such bacteria and thus consequently contributed to the induction of Toll-like receptor 5 (TLR5)-mediated innate immune response (Haiko and Westerlund-Wikström 2013; Hayashi et al. 2001; Neville et al. 2012). Flagella and pili have been observed in motile lactobacilli belonging to *Lactobacillus salivarius* and *Lactobacillus sakei* clades as well as other *Lactobacillus* species as *L. ruminis* strain GRL 1172 (Cousin et al. 2015; Neville et al. 2012; Yu et al. 2017). However, no report regarding flagellated *L plantarum* has been disclosed so far. The presence of flagellin-encoding genes could, therefore, be potentially pivotal factor contributing to highly adhesive property of these infant isolates. This could strongly provide competitive advantage as shown even in the displacement assay, in which the pathogenic bacteria were firstly allowed to occupy mucin-coating surface. Thus, these infant isolates could be considered as the potential probiotic candidate displaying high competition against pathogenic bacteria.

L. plantarum expressed a high level of adhesion mainly derived from intestinal environment, in which host surface attachment was essentially required for their successful colonization. The major adhesion mechanisms seem to be mediated through electrostatic and hydrophobic interactions of cell surface proteins. Multiple mucin-binding protein encoding genes with LPxTG motif detected within the draft genome sequences of *L. plantarum* were quite variable, suggesting that *L. plantarum* could adapt to colonize on many different types of surfaces by modulating expression level of these genes. Interestingly, the mannose-specific adhesion may not always be essential for adhesion to host intestinal mucin. FliC predicted domain located in the LPXTG-anchored protein encoding genes of *L. plantarum* genome could potentially contribute to host adhesion. Its potential role in host-microbe interaction should, therefore, be further elucidated.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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